Impaired Nitric Oxide Synthase Pathway in Diabetes Mellitus
Role of Asymmetric Dimethylarginine and Dimethylarginine Dimethylaminohydrolase

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Background—An endogenous inhibitor of nitric oxide synthase, asymmetric dimethylarginine (ADMA), is elevated in patients with type 2 diabetes mellitus (DM). This study explored the mechanisms by which ADMA becomes elevated in DM.

Methods and Results—Male Sprague-Dawley rats were fed normal chow or high-fat diet (n=5 in each) with moderate streptozotocin injection to induce type 2 DM. Plasma ADMA was elevated in diabetic rats (1.33±0.31 versus 0.48±0.08 μmol/L; P<0.05). The activity, but not the expression, of dimethylarginine dimethylaminohydrolase (DDAH) was reduced in diabetic rats and negatively correlated with their plasma ADMA levels (P<0.05). DDAH activity was significantly reduced in vascular smooth muscle cells and human endothelial cells (HMEC-1) exposed to high glucose (25.5 mmol/L). The impairment of DDAH activity in vascular cells was associated with an accumulation of ADMA and a reduction in generation of cGMP. In human endothelial cells, coincubation with the antioxidant polyethylene glycol–conjugated superoxide dismutase (22 U/mL) reversed the effects of the high-glucose condition on DDAH activity, ADMA accumulation, and cGMP synthesis.

Conclusions—A glucose-induced impairment of DDAH causes ADMA accumulation and may contribute to endothelial vasodilator dysfunction in DM. (Circulation. 2002;106:987-992.)

Key Words: nitric oxide ■ endothelium ■ oxidative stress

Cardiovascular complications are the major cause of mortality and morbidity for the 135 million individuals worldwide afflicted by type 2 diabetes mellitus (DM).1–3 Endothelial dysfunction is a common feature in diabetic patients4–6 and may contribute to cardiovascular morbidity.7–9 Mechanisms of diabetes-induced endothelial dysfunction include the production of prostanoid vasoconstrictors and the increased oxidative degradation of NO.10,11 Deficiency of NO increases vascular resistance and promotes atherosclerosis.12 In addition to its increased oxidative degradation, another possible mechanism for NO deficiency and cardiovascular morbidity is reduced NO synthesis caused by asymmetric dimethylarginine (ADMA).13

ADMA is an endogenous competitive inhibitor of NO synthase (NOS).14 This modified amino acid is derived from proteins that have been posttranslationally methylated and subsequently hydrolyzed.15 ADMA is in part cleared by renal excretion.16 Reduced clearance of ADMA in renal failure is associated with endothelial vasodilator dysfunction, reversible by administration of L-arginine14,17 or by dialysis, which removes plasma ADMA.18 However, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) accounts for most of the clearance of ADMA.19 DDAH metabolizes ADMA to L-citrulline and dimethylamine.20 ADMA is elevated to a level that can significantly inhibit NOS activity in individuals with hypercholesterolemia, hypertension, hyperhomocyst(e)inemia, tobacco exposure, and hyperglycemia.21–23 We have proposed that in each of these conditions, the elevation in ADMA is a result of oxidative stress, which impairs the ability of DDAH to metabolize ADMA.19,21 Hyperglycemia can elevate intracellular oxidative stress through multiple mechanisms.25,26 Accordingly, we tested the hypothesis that hyperglycemia-induced oxidative stress impairs DDAH activity, leading to an elevation of ADMA and inhibition of endothelium-derived synthesis of NO.

Methods

Metabolic Studies
Ten male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) were assigned to either normal chow (control, n=5) or

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high-fat diet (60% fat; Harlan Teklad) and streptozotocin (STZ; 35 mg/kg) injection (non–insulin-dependent diabetes mellitus, NIDDM, n=5). The latter diet, combined with a low dose of STZ (to induce a moderate pancreatic injury), is an established model of NIDDM. After 4 weeks, rats were killed after 12 hours of fasting, and blood was collected for determination of plasma glucose (in mg/dL),28 insulin (in μU/mL),29 triglyceride (in mg/dL),30 and ADMA (in μmol/L) concentrations. These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

**ADMA Measurement**
Concentrations of plasma l-arginine, symmetric DMA (SDMA), and ADMA were measured by high-performance liquid chromatography (HPLC) as previously described,21 with the following modification. A gradient mobile phase, consisting of potassium phosphate buffer (pH 6.5) and HPLC graded methanol, was used. The HPLC process is initiated with a mobile phase containing 15% methanol, which is increased linearly to 25% over the first 20 minutes, and then to 27% over the subsequent 15 minutes. This modification reduces peak broadening, enhances separation, and decreases procedure time.

**Western Blotting**
After removal, abdominal aortas were rapidly rinsed and placed on ice in lysis buffer containing 10 mmol/L sodium phosphate buffer, 1% Igepal CA-630 (Sigma), trypsin inhibitor, leupeptin, pepstatin, and antipain (each 10 mg/L). Tissues were homogenized and centrifuged. Protein concentration of supernatants was measured by Lowry assay. Hybridized chemiluminescence nitrocellulose membranes with proteins transferred from SDS-PAGE gels were incubated with a mouse monoclonal antibody (1:500) against purified human DDAH-I31 in Tris-buffered saline containing 4% nonfat milk and probed with a peroxidase-conjugated anti-mouse antibody (1:1000), detected by enhanced chemiluminescence.

**Cell Culture**
HMEC-1, a human dermal microvascular endothelial cell line,31 was grown in DMEM (Gibco BRL) containing 5.5 mmol/L glucose, 20% FBS, 84 mg/L l-arginine, 110 mg/L sodium pyruvate, 1% glutamine, and 1% gentamicin and incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded at a density of 5000 cells/cm² and grown to 80% confluence (3 to 4 days).

Subconfluent (80%) HMEC-1s were washed twice with PBS (pH 7.0) and cultured in control (5.5 mmol/L d-glucose), osmotic-control (control with 20 mmol/L mannose), or high-glucose (25.5 mmol/L) medium containing 5% FBS, with or without polyethylene glycol–conjugated superoxide dismutase (PEG-SOD; 22 U/mL). Cells and media were harvested after 48 hours for DMA concentrations. DDAH activity was determined in confluent cells. Rat vascular smooth muscle cells (RVSMCs) were isolated from abdominal aortas after 1 hour of incubation in RPMI containing collagenase (2 mg/mL, type IV) and elastase (0.4 mg/mL pancreatic) at 37°C. Cells were then grown in control, osmotic control, or high-glucose medium for 3 passages. DDAH activity and expression were determined on confluence of the third passage. All measurements were performed in triplicate, and cells were counted using a hemocytometer.

**Rat Tissue and Cell DDAH Activity Assay**
DDAH activity was assayed by determining l-citrulline formation in tissue homogenates as previously described.32 As negative controls, tissue homogenates were boiled for 10 minutes to inactivate the enzyme. Background values obtained were subtracted from the experimental data to provide the genuine DDAH activity. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol l-citrulline from ADMA per minute at 37°C.

**Endothelial DDAH Activity Assay**
Hitherto, DDAH activity assay has depended on measuring the formation of l-citrulline. However, DDAH is not the sole source of citrulline. NOS, for instance, also produces citrulline. Accordingly, we circumvented this problem by directly measuring the amount of ADMA metabolized by the enzyme. In an ice bath, cell lysates were divided into 2 groups, and ADMA was added (final concentration 500 μmol/L). To inactivate DDAH, 30% sulfurosalicylic acid was immediately added to 1 experimental group. This group provided a baseline of 0% DDAH activity. The other lysate was incubated at 37°C for 2 hours before the addition of 30% sulfurosalicylic acid. The ADMA level in each group was measured by HPLC as described above. The difference in ADMA concentration between the 2 groups reflected the DDAH activity. For every experiment, DDAH activity of HMEC-1s exposed to normal glucose levels is defined as 100%, and DDAH activities in other conditions were expressed as percentages of the ADMA metabolized compared with the control.

**Determination of cGMP Levels in Endothelial Cells**
Subconfluent HMEC-1s cultured in medium containing normal glucose level (5.5 mmol/L, with 20% FBS) were exposed to the experimental conditions for 48 hours. To assess synthesis of bioactive NO, HMEC-1s were stimulated with calcium ionophore (A23187, 10 μmol/L) after 15 minutes of preincubation with zaprinast (30 μmol/L; a type V phosphodiesterase inhibitor). HMEC-1s were incubated with A23187 and zaprinast for 2 hours, after which the media were aspirated. Intracellular cGMP was assayed by electroimmunoassay according to the protocol provided by the manufacturer (Amersham).

**Statistical Analysis**
Data are expressed as mean±SEM. Comparisons of multiple means were made by ANOVA followed by a Fisher’s protected least significant difference test. Pearson correlation coefficients were calculated when indicated. Comparisons between 2 groups were made by Students’ t test for unpaired observations. Probability values of P<0.05 denote statistical significance.

**Results**

**Metabolic Studies**
Significant increases in both plasma glucose and triglyceride concentrations were observed in the NIDDM rats (glucose: 416±5 versus 170±21 mg/dL in the control; triglyceride: 205±55 versus 92±5.5 mg/dL; P<0.05 in both cases). Insulin levels were not significantly different between the NIDDM and the control rats (32±6.1 versus 29±3.6 μU/dL). Plasma ADMA concentrations were significantly elevated in NIDDM rats (Figure 1).
Aortic DDAH Expression and Activity

No difference in aortic DDAH expression was observed between control and NIDDM rats (Figure 2A). In contrast, aortic DDAH activity was significantly reduced in NIDDM rats (0.05 ± 0.01 versus 0.09 ± 0.01 U/g protein in control, P < 0.05; Figure 2B). In addition, aortic DDAH activity negatively correlated with the levels of plasma ADMA (Figure 3).

DDAH Activity and Expression in Cultured Vascular Cells

Protein expression and activity were assessed in RVSMCs incubated with a normal or a high level of glucose. The high-glucose condition did not change DDAH expression (Figure 4A). However, DDAH activity was significantly reduced in RVSMCs exposed to high glucose (Figure 4B). Like VSMCs, HMEC-1s exposed to high-glucose conditions manifested a reduced DDAH activity (Figure 5). Mannose had no effect on endothelial DDAH activity (osmotic control, Figure 5). Reduction of endothelial DDAH activity in cells exposed to high glucose was reversed by addition of PEG-SOD.

Endothelial ADMA and SDMA Elaboration

The reduction in endothelial DDAH activity was associated with an elevation of ADMA concentration in the medium. Glucose dose-dependently elevated ADMA accumulation over 48 hours. Coincubation with the antioxidant PEG-SOD blocked the effect of glucose on ADMA accumulation (Table). Mannose had no effect on ADMA (1.37 ± 0.17 versus 1.44 ± 0.14 μmol/L, mannose condition versus control, data not shown). SDMA accumulation in the medium was unaf-
Effect of Glucose and/or PEG-SOD on Human Endothelial ADMA Elaboration

<table>
<thead>
<tr>
<th>Glucose Concentration, mmol/L</th>
<th>ADMA (- PEG-SOD)</th>
<th>ADMA (+ PEG-SOD)</th>
<th>SDMA (- PEG-SOD)</th>
<th>SDMA (+ PEG-SOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.44±0.07</td>
<td>1.38±0.15</td>
<td>0.31±0.08</td>
<td>0.35±0.11</td>
</tr>
<tr>
<td>15</td>
<td>1.83±0.11*</td>
<td>1.41±0.09†</td>
<td>0.38±0.12</td>
<td>0.33±0.13</td>
</tr>
<tr>
<td>25</td>
<td>2.21±0.12*</td>
<td>1.32±0.32†</td>
<td>0.36±0.15</td>
<td>0.39±0.09</td>
</tr>
<tr>
<td>30</td>
<td>2.57±0.25*</td>
<td>1.45±0.24†</td>
<td>0.39±0.14</td>
<td>0.41±0.17</td>
</tr>
</tbody>
</table>

Values are expressed as μmol/L ADMA or SDMA per 10^7 cells. *P<0.05 from the control (5 mmol/L glucose); †P<0.05 from corresponding control values (without PEG-SOD).

affected by glucose concentration and was not altered by the addition of PEG-SOD (Table).

Endothelial cGMP Levels

To confirm the pathological relevance of elevated ADMA to the NOS pathway, endothelial cGMP levels were assayed and found to be significantly reduced in cells exposed to high glucose (Figure 6). Mannose did not alter endothelial cGMP level. Addition of PEG-SOD to the high-glucose medium restored cGMP production to a level comparable to that observed during the normal-glucose condition.

Discussion

The salient findings of the present investigation are as follows: (1) plasma ADMA concentrations are elevated in NIDDM rats and are associated with reduced DDAH activity; (2) prolonged exposure to high glucose decreases DDAH activity in cultured endothelial cells or VSMCs; (3) elevated glucose impairs DDAH activity; through this impairment, glucose dose-dependently elevates endothelial ADMA elaboration to reduce nitric oxide synthesis; and (4) these effects of elevated glucose are reversed by the intracellular antioxidant PEG-SOD.

Vascular NO activity is reduced in diabetes, leading to impaired endothelium-dependent vasodilation and elevated platelet aggregation. It is possible that reduced NO activity contributes to the increased cardiovascular morbidity observed in patients with diabetes. Of note, elevated levels of ADMA are predictive of carotid artery disease. Furthermore, 2 recent studies indicate that plasma ADMA is an independent predictor of cardiovascular events and total mortality.

Previous studies have shown that exogenous ADMA in concentrations between 1 and 10 μmol/L dose-dependently reduces endothelium-dependent NO-mediated vasodilation in isolated rat mesenteric vessels and cerebral vessels, inhibits NO production by cultured macrophages, and increases endothelial adhesiveness to monocytes. ADMA elevations in diabetic individuals have been reported previously. Results from the present investigation are consistent with these observations. We found that plasma ADMA concentrations are elevated 3-fold in NIDDM rats. The increase of ADMA may have pathophysiological significance, because its concentration falls into the range shown to inhibit the activity of NOS.

ADMA is generated from the hydrolysis of ubiquitous proteins containing methylated arginine residues. The nuclear protein arginine N-methyltransferase (protein methylase I) has been shown to methylate internal arginine residues in a variety of proteins. The methyl groups may be distributed symmetrically or asymmetrically to the guanidinium nitrogen of arginine, resulting in SDMA, N-monomethylarginine, and ADMA, with ADMA being the predominant isomer. These methylated arginines are excreted in the urine. In addition, the metabolism of ADMA and N-monomethylarginine, but not SDMA, occurs via hydrolytic degradation to l-citrulline and dimethylamine by DDAH.

We recently showed that reduced DDAH activity, but not expression, is responsible for plasma ADMA elevation in hypercholesterolemia and hyperhomocyst(e)inemia. Similarly, in the present study, we found that NIDDM rats manifest reduced DDAH activity in the absence of any change in DDAH expression. DDAH activity of the aorta was significantly decreased in NIDDM rats (Figure 4, A and B). One caveat to note is that there are 2 isoforms of DDAH. Whereas our antibody revealed no change in DDAH-1 expression, it is possible that there was an undetected change in DDAH-2 expression that could explain in part the reduction in DDAH activity.

Diabetes is a constellation of physiological abnormalities. A number of metabolic syndromes probably contribute to endothelial dysfunction. We decided to focus on the specific effect of high glucose on ADMA. The decline in DDAH activity was strongly associated with elevated ADMA levels in the plasma in vivo and in the conditioned medium in vitro. This effect occurred without any change in SDMA, which is not metabolized by DDAH. Hence, elevated glucose raises endothelial elaboration of ADMA by reducing its breakdown (by DDAH), but not by increasing protein methylation.

DDAH is an oxidant-sensitive enzyme. The sulfhydryl group of its cysteine 249 residue participates in the reaction-intermediate formation by its nucleophilic attack of guanidinium carbon of ADMA. Sulphydryl-blocking agents such as p-chloromercuribenzoate and HgCl₂, are known to inhibit the activity of the enzyme. DDAH dysfunction hence seems...
plausible, especially in the setting of DM, in which hyperglycemia has been known to elevate oxidative stress.\(^{25,26}\) Several pathways have been characterized to account for the increased production of free radicals in hyperglycemia. For instance, elevated glucose may activate the polyol pathway, leading to the oxidation of sorbitol to fructose, coupled by the reduction of \(\text{NAD}^+\) to \(\text{NADH}\).\(^{22-24}\) The increased ratio of \(\text{NADH}/\text{NAD}^+\) may in turn promote free-radical production by activating xanthine oxidase and inactivating intracellular and extracellular SOD. It is possible that these processes contribute to reduced DDAH activity.

We hypothesized that glucose-induced oxidative stress would impair DDAH activity. This hypothesis was supported by the observation that the antioxidant PEG-SOD restored DDAH activity in the high-glucose condition, with a corresponding reduction in endothelial ADMA accumulation.

We assessed the effect of high glucose on the NOS pathway by detecting endothelial cGMP production rather than by measuring the levels of NO, using chemiluminescence, because the former better reflects bioactive NO. Measurement of nitrate and nitrite levels by chemiluminescence, because the former better reflects bioactive NO. Although NO metabolites may be increased in diabetes,\(^4\) bioactive NO is reduced,\(^7,8\) as confirmed in this study. Furthermore, we observed that the antioxidant PEG-SOD reversed the effect of high glucose levels on cGMP production. This is not surprising, because the effect of PEG-SOD to scavenge \(\text{O}_2^-\) radicals may enhance cGMP production at 2 levels: (1) by restoring the activity of DDAH and thus lowering ADMA and (2) by reducing the oxidative degradation of NO.

ADMA and DDAH are widely distributed in tissues\(^1,6\) and appear to play a seminal role in regulating NO synthesis in physiological and/or pathological states. In this study, we found that hyperglycemia elevates ADMA by impairing DDAH activity in vascular smooth muscle and the endothelium. The effect of hyperglycemia to impair DDAH activity is probably mediated by oxidative stress. This is a novel mechanism for diabetes-induced endothelial vasodilator dysfunction, which may contribute to the cardiovascular morbidity observed in patients with DM.

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